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Sperm 1: A POU-domain gene transiently expressed immediately before meiosis I in the male germ cell

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ABSTRACT **Members of the POU-domain gene family encode for transcriptional regulatory molecules that are important for terminal differentiation of several organ systems, including anterior pituitary, sensory neurons, and B lymphocytes. We have identified a POU-domain factor, referred to as sperm 1 (Sprm-1). This factor is most related to the transactivator Oct-3/4, which is expressed in the early embryo, primordial germ cells, and the egg. However, in contrast with Oct-3/4, rat Sprm-1 is selectively expressed during a 36- to 48-hr period immediately preceding meiosis I in male germ cells. Although the POU-domain of Sprm-1 is divergent from the POU-domains of Oct-1 and Oct-2, random-site-selection assay reveals that Sprm-1 preferentially binds to a specific variant of the classic octamer DNA-response element in which the optimal sequence differs from that preferred by Oct-1 and Pit-1. These data suggest that the *Sprm-1* gene encodes a DNA-binding protein that may exert a regulatory function in meiotic events that are required for terminal differentiation of the male germ cell.**

Spermatogenesis is a terminal differentiation process whereby male germ cells develop into mature spermatozoa (1, 2). Primordial germ cells, derived from primitive ectoderm, are established in the primitive gonad on embryonic day 10.5–12.5 in the mouse. After birth these cells proliferate extensively giving rise to type A spermatogonia, which can either replicate as stem cells or differentiate to type B spermatogonia. At puberty type B spermatogonia develop into large diploid primary spermatocytes that undergo two reductive divisions, giving rise to the haploid spermatids. Spermatids evolve into mobile spermatozoa through a process referred to as spermiogenesis, characterized by restructuring of their nuclei and development of flagella. An essential component of spermatogenesis is meiosis, a process that involves a single round of DNA replication, pairing, and recombination, followed by two reductive divisions (2). Although meiotic reduction has been extensively studied in yeast (for review, see ref. 3), much less is known of the regulatory factors that may be involved in meiosis in mammals.

Molecular cloning of the mammalian DNA-binding proteins Oct-1, Oct-2, and Pit-1, and the *Caenorhabditis elegans* developmental regulatory gene *unc-86*, revealed that all had a common sequence referred to as the POU-domain, which is required for high-affinity DNA-binding and protein–protein interactions (4). The POU-domain is a bipartite structure composed of the POU-specific domain, which is connected by a short variable linker sequence to the POU homeodomain. Subsequently, several additional members of this gene family, most of which are predominantly expressed in the developing and adult nervous system, have been described in mammals (for review, see refs. 5–7). Notable exceptions to this expression pattern are provided by Oct-1 (8) and Brn-5 transcripts (9), which have widespread distribution; Pit-1, Oct-2, and Skn-1a/i transcripts, which are expressed in the anterior pituitary (10), B lymphocytes (11), and skin (12), respectively; and Oct-3/4 protein, which is expressed in undifferentiated cells early in development and later becomes restricted to oocytes (13–16).

Genetic evidence indicates that Oct-2, Pit-1, and unc-86 proteins are required for cell determination and/or function in B lymphocytes (17), anterior pituitary (18), and sensory neurons (19), respectively. Although the functions of other POU proteins remain elusive, the present evidence suggests that many members of this family may have important roles in cell specification and terminal differentiation. In this report we describe the characterization of a member of the POU-domain family, referred to as sperm 1 (Sprm-1), which is expressed transiently before meiosis I during spermatogenesis in the male germ cell.¶ This highly restricted expression pattern during spermatogenesis suggests a possible role for Sprm-1 in the terminal differentiation of the male germ cell.

MATERIALS AND METHODS

cDNA Cloning. cDNA was generated as described from several rat tissues from adults and embryos (12). This cDNA was used as a template in the PCR with degenerate primers corresponding to the third helix of the POU-specific domain (5′ oligo, 5′-TAGAATTCARWSNACNATHWSNMGNTTYGA-3′) and the third helix of the POU homeodomain (3′ oligo, 5′-TAGGATCCTGNSDNYKRTTRCARAACCANAC-3′). One of the POU-domains identified in this screen, Sprm-1, was used to screen $\approx 10^6$ plaques from a rat testis cDNA library. The testis cDNA was generated using random hexamers and was cloned into λ Zap II as described (12). Four clones from the testis library were sequenced on both strands using the dideoxynucleotide chain-termination method as described (12). The longest clone from the testis library was 1231 bp long, in agreement with the size of the actual transcript in testis, suggesting that the identified cDNA is full length.

mRNA Analyses. RNase protection assays were done as described using 20 μg of total RNA and a ^{32}P-labeled antisense RNA probe composed of nt 349–786 (20). RNA blot hybridization was done with a random-primed ^{32}P-labeled Sprm-1 DNA probe composed of nt 349–786. Four micrograms of poly(A)$^+$ RNA was loaded in each lane. To ensure equal loading the blot was rehybridized with a β-actin probe as described (20). Similar results were obtained when we

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Abbreviations: SAAB, selected and amplified binding; Sprm-1, sperm 1.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L23864).

used a probe corresponding to the NH_2 terminus of Sprm-1 to probe RNA blots (data not shown).

***In Situ* Hybridization.** The rat testes were from 60-day-old animals. *In situ* hybridization studies were done as described (12, 21). The probes were ^{35}S-labeled antisense and sense Sprm-1 RNA probes. At least 10 round tubules at each stage of spermatogenesis were evaluated at ×400 power, and the total number of grains per round tubule was quantified. Results are expressed as a percentage of the tubule with the maximal number of grains. The number of grains over tubules of all stages, except stages IX, XII, and XIII, was the same whether sense or antisense probes were used. Thus, expression of Sprm-1 mRNA was limited to these three indicated stages of the cycle of the seminiferous epithelium.

DNA–Protein Binding Assays. Gel-mobility-shift assays were done as described (12). Sequences of hepatmer-octamer (H^+O^+), octamer (H^-O^+), hepatmer (H^+O^-), herpes simplex octamer (HSV oct), prolactin 1P octamer (pOct), corticotropin-releasing hormone POU site (CRH) homeodomain binding site from *Drosophila ftz* (Ftz) and *ubx* (Ubx) promoters, a POU binding site from the rat P0 promoter (P0), a Pit-1 binding site from the rat prolactin promoter (Prl 1P) and the pro-opiomelanocortin DE2 site (DE2) are as described (12, 22); other sequences are as indicated: engrailed (En), 5′-AAGGGGATCCAAATGTCAATTAAATATCAA-3′; pro-opiomelanocortin (POMC) CE2, 5′-TCCTCATTAGTGATATTTACCTCCAAATGC-3′; interleukin 2 (Il2) Oct, 5′-TTTGAAAATATGTGTAATATGTAAAACATTTTG-3′. Care was taken to ensure that each binding site was labeled to similar specific activity, and an equivalent amount of labeled site was used in each binding reaction. The Sprm-1 protein was a glutathione *S*-transferase fusion protein purified by glutathione-affinity chromatography (23). The Pit-1 protein was bacterially expressed as described (24). The Oct-2 protein was *in vitro* translated as described (12). The *s*elected *a*nd *a*mplified *b*inding site (SAAB) assay was performed as described (25). The sequence of the template was 5′-CGATGAATTCCTAAGCGCATNNNNNNNNGAGCTCAGATCTC-3′ (fixed part of site is underlined). The sequences of the primers that were used for amplification of the template were 5′-CGATGAATTCCTAAG-3′ (sense) and 5′-ACGAGATCTGAGCTC-3′ (antisense). The conditions for the PCR were as follows: 30–35 cycles at 94°C for 45 sec, 48°C for 2 min, and 72°C for 30 sec. The gel-mobility-shift conditions were as described (12). The selected template was sequenced after four rounds of selection by the means of a ^{32}P-labeled sense primer as described (25).

RESULTS AND DISCUSSION

To identify additional developmental regulators of the POU-domain family, we employed a PCR strategy using degenerate oligonucleotides and cDNAs from several tissues. One of the additional POU-domains identified by this approach showed considerable similarity to the *Oct-3/4* gene (Fig. 1*B*; refs. 13–16). Because preliminary experiments had suggested that this factor, referred to as Sprm-1, was highly expressed in testis, the POU-domain was used to isolate four independent clones from a testis cDNA library. The longest cDNA insert contained an initiation codon within a strong Kozak consensus sequence (26) 55 bp from the 5′ end. The 1008-bp-long open reading frame predicted a 335-amino acid translation product with a molecular mass of 37 kDa (Fig. 1*A*). Sprm-1 exhibits considerable homology to Oct-3/4 in the POU-domain and in the COOH terminus (Fig. 1*B*) but exhibits no significant homology in the NH_2 terminus. There was no homology outside the POU-domain to *Oct-3/4*-related genes previously described in *Xenopus* (27, 28). *Sprm-1* is a single-copy gene localized to mouse chromosome 13 (29).

To determine the expression pattern of this gene, we performed RNase protection assays using total RNA from several adult and embryonic tissues, including spleen, muscle, neonatal skin, liver, lung, heart, pituitary, kidney, adrenal, brain, gut, pancreas, placenta, thymus, ovary, and head and bodies from mouse embryos from embryonic days 9.5–17.5 (Fig. 1*C* and data not shown). This analysis revealed a

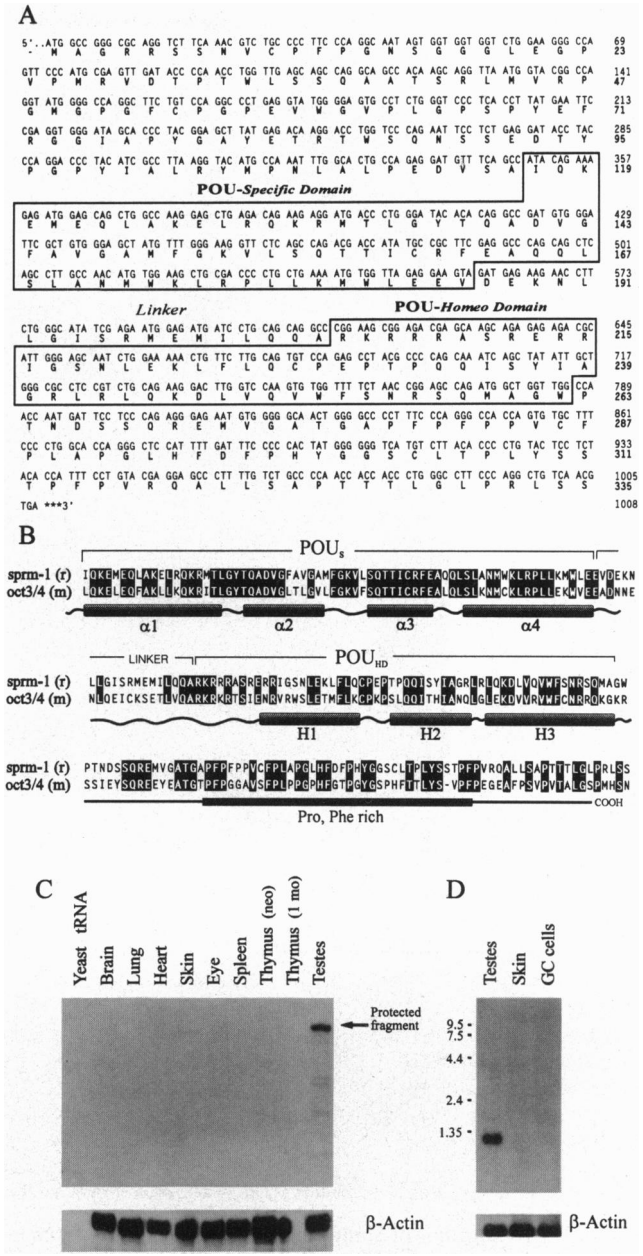

FIG. 1. Sequence and expression of the Sprm-1-encoding gene. (*A*) Nucleotide and deduced amino acid sequence (single-letter code) of Sprm-1. The POU-specific domain and POU homeodomain are boxed. Numbers at right indicate number of nucleotides and amino acids (in boldface type) from the putative initiation codon. (*B*) Comparison of Sprm-1 (r, rat) and Oct-3/4 (m, mouse) proteins; only the homologous regions are shown. Gaps are introduced for maximum alignment, and homology is indicated with reverse highlighting. The proposed structural helices in the POU-specific (POU_s) domain (α 1–α 4) and POU homeodomain (POU_{HD}, H1 to H3) are indicated below. (*C*) RNase protection assays with total RNA from the indicated tissue sources. RNA from thymus was from 4-day-old (neo) and 1-month-old (1 mo) rats. Upper and lower bands show a protected fragment with Sprm-1 (indicated with an arrow) and β-actin probes, respectively. (*D*) RNA blot hybridized with radioactively labeled Sprm-1 (upper band) and β-actin (lower band) DNA probes. The source of RNA is indicated on top, and numbers at left refer to sizes of an RNA ladder (BRL) in kilobases.

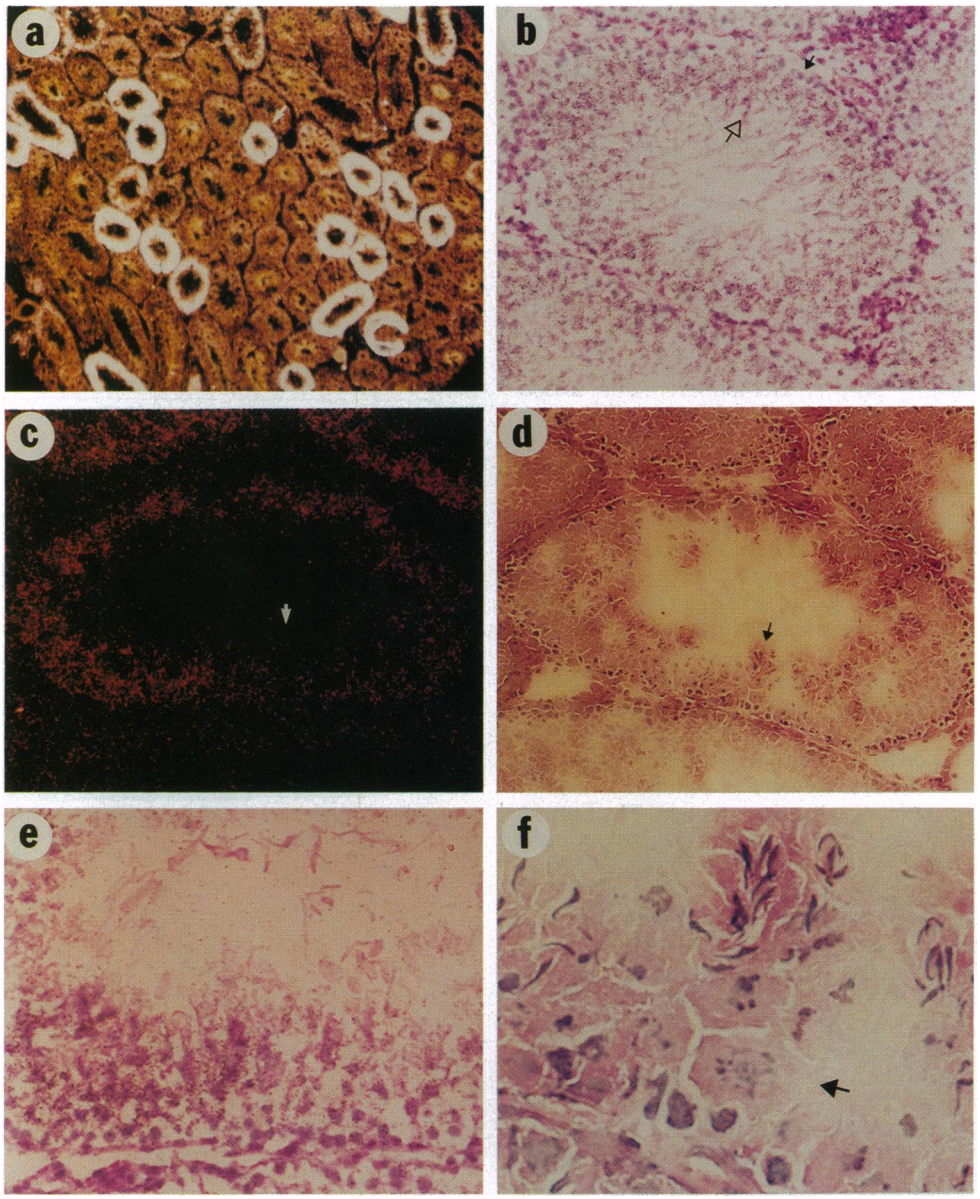

FIG. 2. Localization of Sprm-1 mRNA in adult testis by *in situ* hybridization. (*a*) Low-magnification dark-field view of a cross section through adult testis hybridized with Sprm-1 antisense RNA probe. Arrow, expressing seminiferous tubule. (*b*) High-magnification light-field view of a stage XII seminiferous tubule hybridized with Sprm-1 antisense probe. The filled and open arrows point to areas with spermatogonia and spermatids in acrosome phase, respectively. Note the absence of silver grains over these areas and high-intensity signal over the region between the two arrows that contains primary spermatocytes. (*c*) High-magnification dark-field view of a seminiferous tubule cut at an oblique angle and hybridized with Sprm-1 antisense probe. The white arrow points to an area of decreased signal intensity. (*d*) Bouins' fixed section adjacent to the section of *c*. This seminiferous tubule is predominantly in stage XIII, and the lower right-hand corner of the tubule is progressing toward stage XIV (indicated by arrow). (*e*) High-magnification light-field view of the lower part of the seminiferous tubule shown in *c*. Note fewer silver grains with progression from left to right. (*f*) High magnification of the right lower region of Bouins'-fixed section of *d*. Arrow, Spermatocyte undergoing cell division. (*a*, ×32; *b–d*, ×280; *e*, ×500; *f*, ×1400.)

high level of expression in adult testis. In contrast, we found an extremely low level of expression that was near the detection limit of the protection assay, or was undetectable, in all other tissues examined (Fig. 1*C* and data not shown). Among these tissues, neonatal skin showed the next highest level of expression after testis (Fig. 1*C* and data not shown). No expression was found in retinoic acid-treated or untreated embryonic carcinoma cell lines (P19 and F9; data not shown). Expression in testis was confirmed by an RNA blot with poly(A)$^+$ RNA from adult testis, neonatal skin, and a pituitary somatotroph cell line (GC). A single 1.2-kb transcript was detected in testis, whereas no signal was found in skin, consistent with our estimate that expression in testis is at least 50- to 100-fold higher than in skin (Fig. 1*D*). In addition, no expression was detected by *in situ* hybridization using 16.5- and 17.5-day-old rat embryos (data not shown). We therefore conclude that expression of the Sprm-1-encoding gene is highly restricted to adult testis.

During spermatogenesis in the adult rat, germ-cell differentiation advances in highly ordered waves along the long axes of the seminiferous tubule (1, 2). Therefore, each cross section of a seminiferous tubule contains a series of concentric cohorts of developing germ cells within which all are at the same developmental stage. This arrangement permits classification of seminiferous tubules into 14 stages on the basis of the characteristic morphological appearance and cellular associations of spermatogonia, spermatocytes, and spermatids in each stage (1). We used *in situ* hybridization,

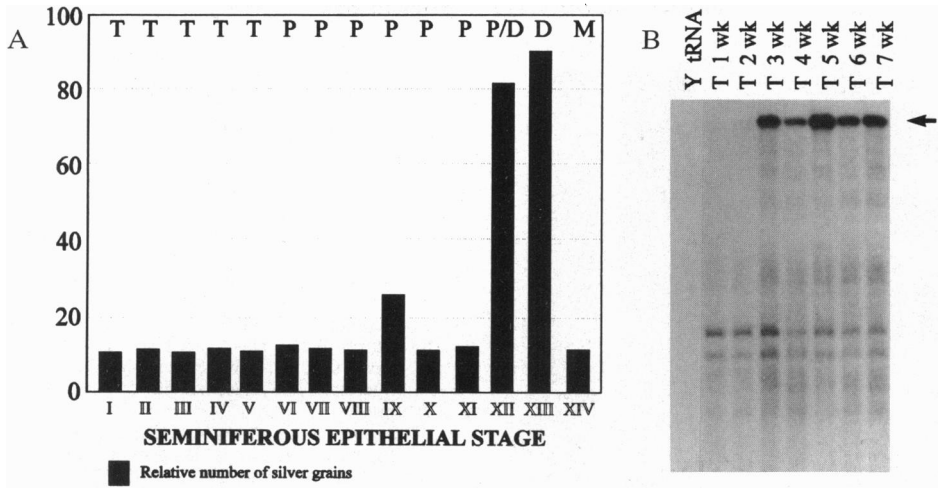

FIG. 3. Stage-specific expression of Sprm-1 mRNA in testis. (*A*) Arbitrary quantitation of Sprm-1 mRNA in seminiferous tubules at different stages. Roman numerals at bottom refer to the stage of the cycle of the seminiferous epithelium. Letters at top refer to differentiation stage of primary spermatocytes in each cycle stage of seminiferous epithelium. T, transition form; P, pachytene; D, diplotene; M, metaphase. (*B*) Developmental expression of Sprm-1 mRNA as measured by RNase protection assays. Rat testis RNA (T) from animals of the indicated ages (in weeks, wk) was hybridized with Sprm-1 antisense probe. Arrow on right indicates the protected fragment. Y tRNA, yeast tRNA.

combined with Bouins' fixation and staining of adjacent sections of adult testis, to identify the cell type and the stages of spermatogenesis in which Sprm-1 mRNA was expressed. Expression was limited to a subset (10–20%) of seminiferous tubules within the testis (Fig. 2*a*), and in positive seminiferous tubules Sprm-1 mRNA was localized to primary spermatocytes (Fig. 2*b*). Dark-field (Fig. 2*c*) and light-field (Fig. 2*e*) views of an obliquely cut seminiferous tubule demonstrate intense signal, except for a markedly decreased number of silver grains in the lower right corner. This tubule is predominantly in stage XIII, except for the lower right region, which is progressing toward stage XIV. A Bouins'-fixed slide from an adjacent section (Fig. 2*d*) is shown as a reference. Fig. 2*f* is a higher magnification from the lower right region of the Bouins'-fixed section and shows two spermatocytes in the center undergoing cell division, consistent with stage XIV classification. On the basis of analyses of many sections, Sprm-1 mRNA expression was almost entirely limited to seminiferous tubule stages XII and XIII (Fig. 3*A*). The only exception was a rare expression pattern in stage IX tubules in which Sprm-1 signal was localized in association with residual bodies along the basal region of seminiferous tubules near Sertoli cells (Fig. 3*A* and data not shown). No expression was seen in spermatogonia, secondary spermatocytes, spermatids, Leydig cells, macrophages, or other interstitial cells. No signal was detected by using sense Sprm-1 RNA probes (data not shown).

On the basis of these experiments, we conclude that Sprm-1 is expressed predominantly in primary spermatocytes during the 36- to 48-hr period of progression through late pachytene and diplotene stages (stages XII and XIII of the cycle of the seminiferous epithelium). These stages are the final steps in germ-cell differentiation before meiosis I (Fig. 3*A*). This is an unusual pattern of expression because with few exceptions (30) known transcription factors in male germ cells are predominantly expressed in the postmeiotic phases of spermatogenesis (31). Results of RNase protection assays with total RNA from rat testes during the first round of spermatogenesis are consistent with the data obtained by *in situ* hybridization. The onset of Sprm-1 mRNA expression was seen between the second and third week (Fig. 3*B*), correlating with the onset of meiosis during pubertal development.

POU-domain transcription factors are characterized by a bipartite DNA-binding domain composed of a POU-specific domain linked with variable spacing to the POU homeodomain. Studies of the binding characteristics of Oct-1 and Oct-2 POU-domains have shown that both bind with highest affinity to a sequence containing a core octamer element: 5′-ATGC(A/T)AAT-3′ (32–38), with the POU-specific domain and POU homeodomain contacting the left and right half of the site, respectively (34, 35, 38). Because Oct-3/4 protein binds the octamer element with lower affinity than Oct-1 and Oct-2 proteins (37), we tested whether Sprm-1 might bind with higher affinity to a series of sites distinct from the octamer site. As controls we tested binding of these same sites to Oct-2 and Pit-1 proteins (Fig. 4*A*). Sprm-1 has a distinct, although overlapping, binding preference (HSV Oct > CRH > POMC DE2 > Ftz = H^+O^+ > pOct = H^-O^+ sites) compared with Oct-2 (H^+O^+ = H^-O^+ > pOct > Il2 Oct > HSV Oct > CRH) and Pit-1 (H^+O^+ > H^-O^+ = HSV Oct = Prl 1P = Ftz > CRH sites). Alignment of the highest-affinity binding sites for Sprm-1 allowed us to derive a consensus sequence: 5′-GCATNN(±N)TAAT-3′ (Fig. 4*A*). Mutational analyses of the CRH site gave further support for these analyses because mutations in the GCAT and TAAT regions completely inhibited binding, whereas mutations in the variable region had less effect (Fig. 4*B*). These experiments suggest that Sprm-1 has a preference for sites that are distinct from a classic octamer site. To test this hypothesis we used the SAAB assay (25) to identify a preferred binding site for Sprm-1. With this assay we identified a preferred site: 5′-GCA**TATGTTAAT**-3′ (selected nucleotides are in boldface type; Fig. 4 *C* and *D*), which is essentially a variant octamer site. This site is highly related to the preferred binding sites for Oct-1 (38) and Brn-5 (9) proteins. However, because Sprm-1 binds with lower affinity to the immunoglobulin octamer site (H^-O^+) than the selected site (Fig. 4*A* and data not shown), minor differences in the octamer site and the nucleotides surrounding the core octamer motif appear important for selective high-affinity binding of this POU-domain protein (Fig. 4*D*; see also refs. 32 and 36).

Our data, in conjunction with previous studies demonstrating expression of Oct-3/4 gene in primordial germ cells and in the egg (13–16), of Oct-2d transcript in spermatids (39), and of XLPOU-60 gene in *Xenopus* oocytes (27, 28), imply a role for POU proteins at multiple steps in male and female germ-cell differentiation. The highly constrained expression of Sprm-1 immediately before meiosis I suggests a possible role for this factor in meiotic events. It is tempting to

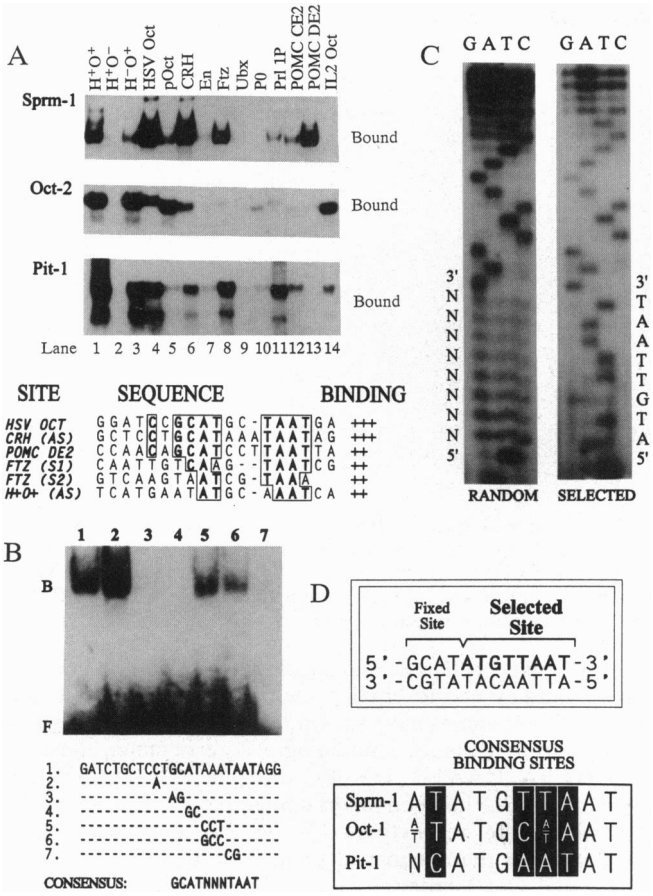

FIG. 4. Specific DNA binding of Sprm-1. (*A*) Binding of Sprm-1 (*Top*), Oct-2 (*Middle*), and Pit-1 (*Bottom*) proteins to indicated sites (shown on top; see DNA–protein binding assays), as measured by gel-mobility-shift assays; only bound complexes are shown. Relative binding affinity to different sites can only be assessed for each protein, and affinity of two different proteins for the same site cannot be compared in this experiment. Alignment of the highest affinity Sprm-1-binding sites is shown below. Ftz(S1) and Ftz(S2) refer to two possible alignments of the homeodomain binding site from the *ftz* promoter (Ftz) site. (*B*) Binding of Sprm-1 to the corticotropin-releasing hormone POU site (CRH) and mutant sites as measured by the gel-mobility-shift assay. Each site was assigned a number as shown at bottom. Bound complexes (B) and free probe (F) are indicated. (*C*) SAAB site for Sprm-1. Sequence of the starting template (on left) is compared with sequence of the template selected by binding with Sprm-1 (on right). Lanes for G, A, T, and C termination reactions are shown. (*D Upper*) The fixed region and selected binding site (in boldface type) for Sprm-1 are shown. (*Lower*) Comparison of the preferred site for Sprm-1, Oct-1 (38), and Pit-1 proteins with variable regions highlighted. Pit-1 consensus binding site was derived by aligning high-affinity sites from the growth hormone, prolactin, and Pit-1-encoding genes. Note that Pit-1 can also bind with high affinity to certain octamer sites (37).

speculate that Sprm-1 might be involved in regulation of genes such as the testis-specific histones that contain octamer elements in their regulatory region (40) and are activated before meiosis in the male germ cell (41). However, we cannot rule out the possibility that Sperm-1 protein is stored in the developing germ cell and serves a function at a later stage of spermatogenesis.

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